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**14. ABSTRACT** Even though prostate cancer is the second leading cause of cancer related mortality in men in the United States, there is an ongoing concern that as a medical community we are over diagnosing, and hence over treating, the disease. Yet, patients at high-risk for metastatic progression are unfortunately treated too late. We hypothesized that tissue chemokines can be strong biomarker candidates for distinguishing patients with high risk for biochemical recurrence or metastatic progression of prostate cancer. Interestingly, the chemokine, CCL4, up regulated in patients with biochemical recurrence. In the past funding cycle, of which we had 4 months of funding due to a lab move, we demonstrated that neutralizing CCL4 reduced the proliferation of mouse prostate andenocarcinoma cells, TRAMP-C1. Further, CCL4 neutralization reduced cell adhesion to collagen I. Orthotopic grafting of TRAMP-C1 cells with prostatic stromal fibroblasts that expressed CCL4 had significantly larger tumors than the TRAMP-C1 tumors associated with vector control expressing stromal fibroblastic cells. Together the data suggested that the upregulation of CCL4 in patient prostatic tissues associated with tumor recurrence is biologically consequential to tumor progression.

**15. SUBJECT TERMS**

None provided.

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**Progress Report**: Regulation and Function of Cytokines That Predict Prostate Cancer Metastasis

a. **INTRODUCTION**

There is a large disparity between the number of newly diagnosed cases of prostate cancer in the United States every year and the number of men who die of the disease. The 30,000 deaths annually in the US caused by prostate cancer are almost entirely due to metastatic progression [1]. As a consequence, even though prostate cancer is the second leading cause of cancer related mortality in men in the United States, there is an ongoing concern that as a medical community we are over diagnosing, and hence over treating, the disease. *Yet, patients at high-risk for metastatic progression are unfortunately treated too late*. The challenge has been to determine up-front which patients harbor high-risk disease requiring aggressive/curative therapy and which patients harbor indolent disease that could be managed with active surveillance. The issue is an important one given the potential for attempts at local curative therapy (whether it be surgery, radiation or cryotherapy) to subject the patient to both short-term and long-term morbidity. Currently clinicians rely on a combinatorial assessment of the pre-treatment PSA value, clinical tumor stage, and biopsy-Gleason score to risk stratify patients. These methods are unable to distinguish 80% of the patients that may not have any clinical consequences from the prostate cancer [2]. Next, following local curative therapy the issue of requirement and timing for second line adjuvant therapy becomes increasing important. However, treatment of cancers prior to metastatic progression with conventional chemotherapy has shown promise of late [3,4,5]. It is critical to accurately determine the appropriate candidates for such adjuvant therapy given the potential for decreased quality of life and added morbidity associated with chemotherapy treatment, especially since the majority (65%) of patients remain disease free after prostatectomy. Since men experiencing PSA recurrence following surgical treatment suggest metastatic spread of the disease, better forms of early detection and risk stratification would support targeted use of adjuvant therapies [6]. Similar to the clinical stratification described for patients prior to primary treatment for prostate cancer, pathologic risk of biochemical recurrence is performed following primary treatment. One of the most commonly used nomogram for post-operative predictions has been described by Kattan and colleagues [7,8]. Multiple criteria that include the pre-treatment PSA, prostate capsule invasion, pathologic Gleason score, surgical margin status, seminal vesicle involvement, and lymph node involvement for predicting post-operative biochemical recurrence [7,8]. However, a model of such clinical/pathologic parameters is limited (particularly at the level of sensitivity) by the fact we do not know all the predictive factors.

Chemokines, cytokines, and growth factors in the tumor microenvironment regulate the fate of tumor progression [9,10]. We hypothesized that tissue chemokines can be strong biomarker candidates for distinguishing patients with high risk for biochemical recurrence or metastatic progression of prostate cancer. CX3CL1 exhibited the best prediction ability ($P < 0.0001$) followed by CCL4 ($P < 0.001$) and IL-15 ($P = 0.003$). The proportional hazard assumption was tested with scaled Schoenfeld residuals [11]. There was no evidence of violation as the chi-square tests for trend were not significant for any of the seven variables (surgical margin status, seminal vesicle involvement, Gleason Score, pre-operative PSA, CCL4, and CX3CL1, and IL-15; P values ranging 0.46 to 0.90). The same two chemokines, CCL4 ($P=0.040$) and CX3CL1 ($P<0.0001$) were significant factors. In addition, pre-operative PSA ($P= 0.0025$) and surgical margin ($P= 0.023$) were significant [12]. We described a strong predictive ability of differentially expressed chemokines in a nested case-control study of prostate cancer patients that develop biochemical recurrence or lead recurrent-free lives following prostatectomy. The goal of this proposal is to determine the biologic role of these potentially clinically relevant chemokines in prostate cancer progression.

Since chemokines regulate BMDC recruitment to tissues, we studied bone marrow derived cells (BMDC) function in the prostate during regrowth. Tissue remodeling, and cancer progression are generally associated with the recruitment of bone marrow derived cells [13]. Co-expression of prostate markers with BMDCs suggested that these recruited cells were also incorporated into the prostate epithelia [14]. We further identified MSCs fusing with prostatic epithelia. Interestingly, the chemokine, CCL4, up regulated in patients with biochemical recurrence, recruits MSC and CX3CL1, down regulated in the recurrent population, is critical to the communication with MSC in eliciting anti-tumor activity. Using an orthotopic human C4-2B xenograft model system, we found that recruited MSCs could further contribute to tumor progression [14]. Ultimately, better understanding of the mechanism of action of the novel set of chemokines impact on cancer metastasis would determine if the chemokines can be therapeutic targets in addition to indicators of a metastatic process.

Due to the move of the lab to Cedars-Sinai Medical Center and funding gap resulting from the grant transfer process, 4 months of funding is represented in this progress report period.

b. **BODY**

The data described here is work done as a result of this DOD award. The focus of **Aim 1** was to determine the effect of the differentially expressed chemokines on a cell autonomous manner on prostate cancer cells. Our knowledge of the importance of the tumor microenvironment in prostate cancer metastatic progression is addressed in **Aim 2** of the proposal. In the last funding period we made significant progress in Aim 1. In the 4 months of funding of the past year we were able to make some progress in better understanding the biologic role of CCL4 in prostate cancer progression through the development of in vitro and in vivo models.

Of the three chemokines: CX3CL1, CCL4, and IL-15, that helped distinguish prostate cancer patients that developed recurrent disease [12], we chose to first examine the role of CCL4. CCL4 was up regulated in patients with recurrent disease. Previously we identified the role of CCL4 upon its overexpression in PC3, C42B, and LNCaP cells. CCL4 seemed to have a role in the proliferation of the PCa lines - but the effect was not statistically significant. However, the CCL4 expression in PCa in men and mice is normally not attributed to as a cancer cell derived factor. Rather, it is expressed by stromal cells [14]. Thus, we overexpressed CCL4 in wild type primary mouse (C57BL/6) prostatic fibroblastic cells, through lenti viral infection. Then the CCL4 expression was verified by RT-PCR, as reliable antibodies for Western blotting are not available.

*In vitro* studies (Aim 1): The conditioned media from wild type prostatic stromal fibroblastic cells that expressed CCL4 and those that expressed the GFP control vector was transferred to TRAMP-C1 cells in 60 mm dishes. Sequential cell counting was performed for 3 days to determine the role of stromally derived CCL4. The results suggested, again a trend of elevated TRAMP-C1 cell proliferation with CCL4, but not statistically significant (**Figure 1**).

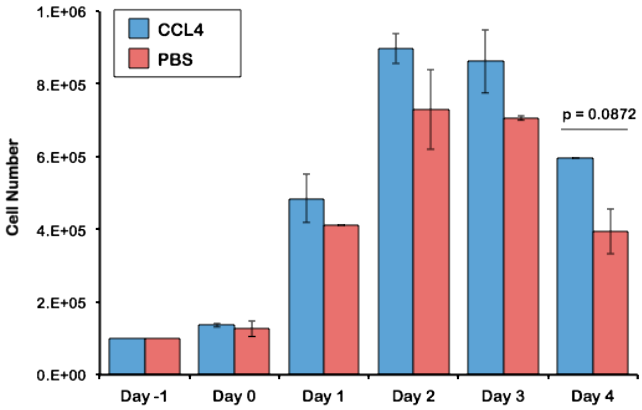


**Figure 1. Addition of CCL4 May be Associated With Increased Proliferation of TRAMP-C1 Cells.** Recombinant CCL4 was added to unconditioned TRAMP-C1 media with 2% FBS. Proliferation was measured by sequential counting over a 96 h period. Data represents one experiment carried out in duplicate.

Then we rationalized that numerous paracrine factors in the carcinoma associated stroma of men and Tgfbr2$^{ColTKO}$ mice are responsible for the tumorigenic phenotype [12,15]; a single factor may be necessary but not sufficient to mediate the differences in tumor progression observed. Thus, we performed conditioned media experiments, transferring media from wild type and Tgfbr2$^{ColTKO}$ prostatic stromal cells in the presence and absence of CCL4 neutralizing antibody (R&D systems). Sequential cell counting was performed in TRAMP-C1 cells. We found a striking proliferative down regulation in the presence of the CCL4 neutralizing antibody compared to IgG isotype control (p value < 0.05) (**Figure 2**). Similarly, we performed adhesion assays with TRAMP-C1 cells on collagen coated plates in the presence and absence of CCL4 neutralizing antibody. We found that neutralization of CCL4 specifically reduced TRAMP-C1 adhesion compared to IgG control at 6-8 µg/well collagen I (**Figure 3).** Interestingly, as the concentration of collagen was elevated to 10 µg/well, the adhesion of the cells was elevated to a point that was not affected by the presence of CCL4 neutralizing antibody.

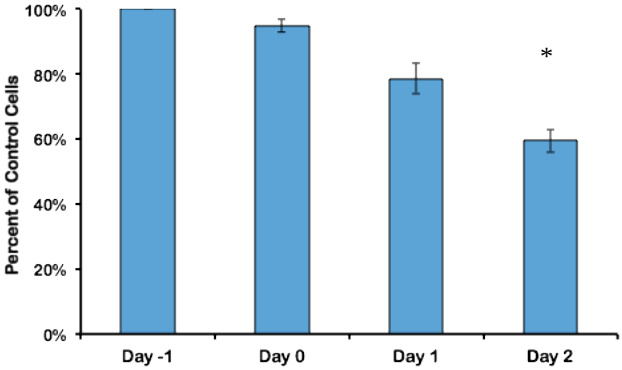


**Figure 2. Neutralization of CCL4 is Associated With Decreased Proliferation of TRAMP-C1 Cells.** Values are presented as means ± SEM and are presented as the percentage of control cells that received conditioned media and an IgG antibody. (* P<0.05 vs. IgG; ANOVA and Tukey's multiple comparison test). Data represents two experiment carried out in triplicate.

Importantly, the mouse TRAMP-C1 cells were used in the studies above, as opposed to the originally proposed human PC3, LNCaP, and C42B cells. Although derived from prostate of C57BL/6 TRAMP mice, express no T-antigen and are not neuroendocrine in differentiation, instead form adenocarcionoma in mice. The reason for the switch in cells lines for the following studies was that we would like to perform in vivo studies in immuno-

competent C57BL/6 mice. The chemokine markers all have obvious roles on immune cells. While the a goal of the proposal is to determine the direct role of the chemokines on PCa epithelia, it would be short sighted to ignore the role of these predictive chemokines on immune cells that also clearly affect cancer progression. The intension is to examine the human cell lines originally proposed as well as the two mouse PCa cell lines available to us now (TRAMP-C1 and MPC3 [a PTEN/p53 deficient adenocarcinoma line recently developed]) in the proposed in vitro and in vivo experiments.

*In vivo* studies (Aim 2): Since the in vivo studies understandably require more time, they were initiated as soon as the stable fibroblastic lines were verified expressing CCL4 or the GFP vector control. The fibroblasts were recombined with TRAMP-C1 cells at a ratio of 3:1 and grafted orthotopically in syngenic C57BL/6 male mice. The tumors were allowed to grow for the next 2 months. The results of this experiment are still being evaluated histochemically, however, grossly the tumors with CCL4 over expressed in the stroma had were approximately 3 fold greater in tumor volume than those tumors having the vector control (**Figure 4**).

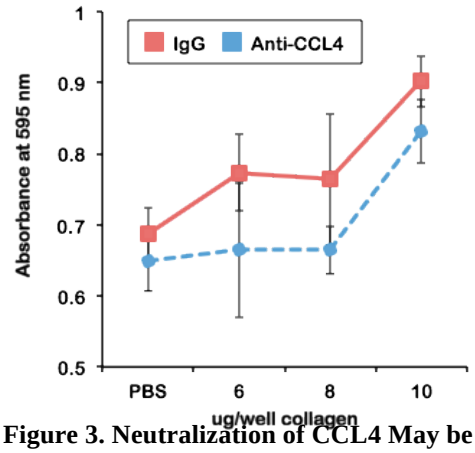


**Figure 3. Neutralization of CCL4 May be Associated With Decreased Adhesion of TRAMP-C1 Cells.**

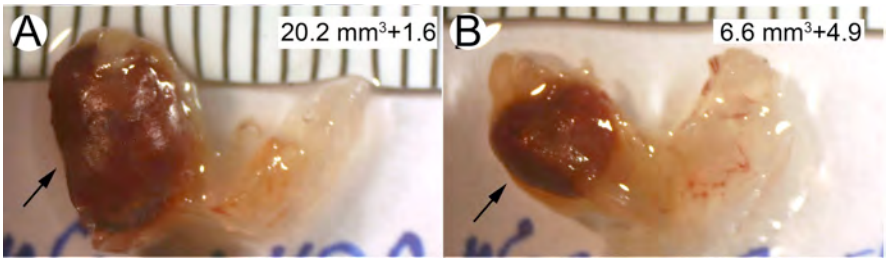


**Figure 4. CCL4 expression causes an increase in tumor growth in orthotopically grafted tissue recombinants.** CCL4 expression by prostatic stromal cells caused a 3-fold increase in tumor size (A) over those expressing GFP control (B).

## c. KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that CCL4 over expression can result in greater proliferation of the TRAMP-C1 mouse prostate cancer epithelial cell line.
- The neutralization of CCL4 has a greater impact on TRAMP-C1 proliferation, in terms of its down regulation than over expression has on its elevation in proliferation.
- CCL4 neutralization increased the adhesion of TRAMP-C1 cells on collagen I.
- The up regulation of CCL4 by prostatic fibroblastic cells increased TRAMP-C1 tumor size in immuno-competent mice with orthotopic allografts.

## d. REPORTABLE OUTCOMES

Research

*Publication*

none.

*Awards received based on work supported by this grant*

- Prostate Cancer Foundation Challenge Award

Products

*CDNA construct, cell lines, and animal models developed*

- Generated CCL4 over expressing mouse prostatic fibroblastic cells

e. **CONCLUSION**

The identification of CX3CL1, IL-15, and CCL4 as differentially expressed chemokines used to predict biochemical recurrence following prostatectomy supported the proposed studies where by CX3CL1 and IL-5 expression was associated with recurrent-free survival, where as CCL4 expression was associated with recurrence [12]. Thus the direct effects of CCL4 of cancer epithelia of varying metastatic potential were tested. We found that CCL4 overexpression in TRAMP-C1 resulted in greater proliferation. However, the neutralization of CCL4 had a greater effect on the proliferation of the prostate cancer epithelia. The proliferative down regulation mediated by the neutralization of CCL4 in media from $Tgfbr2^{ColTKO}$ prostatic stromal cells suggests that CCL4 is necessary for PCa proliferation, but not necessarily sufficient. Thus, the loss in TGF-ß signaling in the stroma of men with prostate cancer can have a proliferative impact on the adjacent epithelia, where CCL4 expression may play a part. Interestingly, CCL4 can also cause greater adhesion of the TRAMPC-1 cells. The apparent greater adhesion could suggest greater motility. However, We did not perform any motility or invasion studies to test this hypothesis yet. Early in vivo studies predictively resulted in greater tumor size when CCL4 was overexpressed by the prostatic stromal cells in tissue recombination allograft studies.

The recent move to Cedars-Sinai Medical Center resulted in a delay in material progress on the proposal. However, the move was explicitly made to take advantage of the exceptional prostate cancer clinical and basic research program to ultimately translate the biologic finding from these biomarkers to prospective clinical trials. The work on the proposal thus far would suggest that at least CCL4 is a biomarker that has biologic significance. This would support our hypothesis that markers with biologic observations are more likely to have success in the clinic in predicting physiologic progression.

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Associate Professor of Medicine

September 1, 2011

ATTN: MCMR-RMI-S
540 Scott Street
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Dear Sir or Madam:

Thank you for the opportunity to share my accomplishments in my training for the past year and the entire fellowship term. I am providing my annual summary report for award number W81XWH-09-1-0503 for the period July 1, 2010 – August 1, 2011. Additionally, per your request I am forwarding my current contact information below. Please contact me if further clarification or additional material is required.

Neil A. Bhowmick, Ph.D.
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Uro-Oncology Research Program
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Please note the grant has been transferred to this institution as of April 12, 2011. There was a gap in funding from July 31, 2010 to April 12, 2011 due to the transfer process. We are currently performing the tasks of the proposal at Cedars-Sinai Medical Center. Thank you.

Sincerely,

Neil Bhowmick, Ph.D.